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# Ibotenic acid and thioibotenic acid: a remarkable difference in activity at group III metabotropic glutamate receptors

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**Abstract**

In this study, we have determined and compared the pharmacological profiles of ibotenic acid and its isothiazole analogue thioibotenic acid at native rat ionotropic glutamate (iGlu) receptors and at recombinant rat metabotropic glutamate (mGlu) receptors expressed in mammalian cell lines. Thioibotenic acid has a distinct pharmacological profile at group III mGlu receptors compared with the closely structurally related ibotenic acid; the former is a potent (low μm) agonist, whereas the latter is inactive. By comparing the conformational energy profiles of ibotenic and thioibotenic acid with the conformations preferred by the ligands upon docking to $mGlu_1$ and models of the other mGlu subtypes, we propose that unlike other subtypes, group III mGlu receptor binding sites require a ligand conformation at an energy level which is prohibitively expensive for ibotenic acid, but not for thioibotenic acid. These studies demonstrate how subtle differences in chemical structures can result in profound differences in pharmacological activity.


*Keywords:* Ibotenic acid; Thioibotenic acid; Glutamate receptor; mGlu receptor; Group III agonist; Docking; Homology model; Conformational energy

## 1. Introduction

(*S*)-Glutamic acid (glu) exerts its function as the major excitatory neurotransmitter in the brain via two distinct classes of receptors: the ionotropic glutamate (iGlu) receptors which belong to a family of ligand-gated ion channels, and the metabotropic glutamate (mGlu) receptors which are members of the G-protein-coupled receptor superfamily (Bräuner-Osborne et al., 2000; Dingledine et al., 1999). So far, 16 iGlu receptor subunits and 8 mGlu receptors have been identified in humans, where they are involved in many physiological processes such as learning, memory, vision, control of movement and pain sensitivity (Gerlai et al., 1998; Holscher et al., 1999; Lu et al., 1997). Malfunctioning glutamatergic pathways have been implicated in a variety of neuropathologies, including epilepsy, stroke, cognitive disorders and neurodegenerative diseases (Bräuner-Osborne et al., 2000; Dingledine et al., 1999; Holscher et al., 1999).

The iGlu receptors are further subdivided into three groups named after the selective agonists *N*-methyl-D-aspartic acid (NMDA), (*RS*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) and kainic acid (Bräuner-Osborne et al., 2000; Dingledine et al., 1999). Likewise, mGlu receptors have been subdivided into three groups based on their sequence homology, signal transduction mechanisms and pharmacological properties (Conn and Pin, 1997; Nakanishi, 1992). Group I consists of $mGlu_1$ and $mGlu_5$, which are coupled to the hydrolysis of phosphatidylinositol. The $mGlu_2$ and $mGlu_3$ receptors constitute group II and are coupled to inhibition of cyclic AMP formation. Group III comprises $mGlu_4$, $mGlu_6$, $mGlu_7$ and $mGlu_8$, which are also negatively linked to adenylyl cyclase activity (Conn and Pin, 1997).

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Ibotenic acid ((*RS*)-2-amino-(3-hydroxy-5-isoxazolyl)-acetic acid) is a naturally occurring excitotoxin, originally isolated from *Amanita muscaria* (Krogsgaard-Larsen and Hansen, 1992). It has been used extensively as a pharmacological and neurotoxicological tool on account of its agonistic effects at iGlu receptors. For example, ibotenic acid has been used to create lesions in the hippocampus (Jarrard, 2002) and other brain regions, in order to investigate physiological and behavioral effects (Lacroix et al., 2000; Lu et al., 2001; Turner et al., 1992). These kinds of brain lesions are also used as models for neurodegenerative diseases, traumatic brain injury and stroke (Eijkenboom et al., 2000).

We have previously demonstrated that the 3-hydroxyisoxazole moiety of ibotenic acid (Fig. 1) can serve as a versatile bioisostere for the distal carboxylic acid function of glu (Bischoff et al., 1995; Krogsgaard-Larsen et al., 1980; Madsen et al., 2001). Substituting sulfur for the ring oxygen creates a parental 3-hydroxyisothiazole system, which is less acidic ($pK_a \sim 7$) and more lipophilic than the 3-hydroxyisoxazole group ($pK_a \sim 5$). There are accompanying subtle changes in molecular geometry, and a 15% increase in the molecular van der Waals volume of the sulfur-containing ring system compared to the corresponding 3-hydroxyisoxazole (Frydenvang et al., 1997). On this basis, we synthesised thioibotenic acid (Fig. 1), the isothiazole analogue of ibotenic acid (Bunch et al., 2002).

We have previously made similar thio analogues of AMPA and AMPA-derived isoxazole amino acids (Matzen et al., 1997a,b). From the pharmacological characterisation of these compounds, no simple correlation between structure and activity was apparent. The oxygen and sulfur analogues have generally shown similar selectivity, but some differences in affinity/potency have been observed.

In the current study, we have determined the pharmacological profile of ibotenic acid and its sulfur analogue thioibotenic acid on both native iGlu and cloned mGlu receptors. Our studies show that replacement of the 3-hydroxyisoxazole ring oxygen by sulfur has a profound effect on the pharmacological activity at group III mGlu receptors, and only minor effects at other subtypes.

The conformation of ibotenic acid has previously been studied by crystallography and Hartree–Fock gas phase calculations (Brehm et al., 1998), but thioibotenic acid and isothiazoles are in general less well-characterised. With the advent of Density Functional Theory and ab initio solvation models capable of addressing the conformational behavior of such highly charged heterocyclic ligands, and with the X-ray crystal structure of the ligand binding domain of the $mGlu_1$ receptor in hand (Kunishima et al., 2000), we are able to propose a structural explanation for this dramatic difference in pharmacological profiles at the group III mGlu receptors.

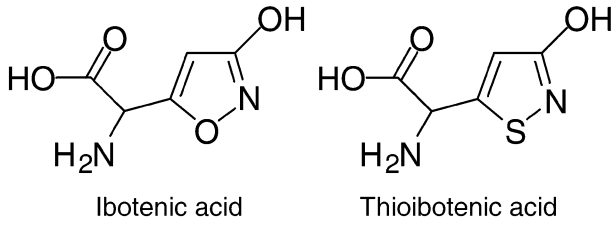


Fig. 1. Structures of ibotenic acid and thioibotenic acid showing the replacement of oxygen with sulfur in the heterocyclic ring of thioibotenic acid.

## 2. Materials and methods

### 2.1. Materials

Thioibotenic acid was synthesised in our laboratory as previously described (Bunch et al., 2002). (*S*)-2-Amino-4-phosphonobutyric acid (L-AP4), [$^3$H]-L-AP4 (45.5 Ci/mmol), ibotenic acid and L-serine-*O*-phosphate (L-SOP) were purchased from Tocris Cookson (Bristol, UK). [$^3$H]AMPA (55.5 Ci/mmol), [$^3$H]kainic acid (58.0 Ci/mmol) and [$^3$H]- (*RS*)-(*E*)-2-amino-4-phosphonomethyl-3-heptenoic acid (CGP39653) (50.0 Ci/mmol) were purchased from NEN (Boston, MA, USA). All other compounds and reagents were from Sigma-Aldrich (Munich, Germany).

### 2.2. Generation of expression construct

The expression constructs for the rat $mGlu_{4a}$, $mGlu_6$ and $mGlu_{8a}$ were subcloned into a modified pEGFP-N1 vector (BD Biosciences, San Jose, USA) essentially as previously described (Pagano et al., 2001). The N-terminal signal peptides for $mGlu_{4a}$, $_6$ and $_{8a}$ were replaced by the $mGlu_5$ signal peptide, because the latter is known to promote a good receptor expression and proper release of the epitope tag (Pagano et al., 2001). To allow detection of receptor surface expression by immunofluorescence a c-myc epitope tag and a unique *Mlu*I site were inserted at the mGlu receptor N-terminal. The DNA coding for green fluorescent protein (GFP) was excised by *Mlu*I/*Not*I digestion and replaced by DNA encoding mGlu receptors subcloned by polymerase chain reaction (PCR). The primers used for PCR, introducing a *Mlu*I/*Not*I site, were as follows: $mGlu_4$ upstream (5′ -CGC GCG ACG CGT AAG CCC AAG GGT CAC CCC CAC ATG-3′ ), $mGlu_4$ downstream (5′ -GCG GCC GCG CGG CCG CCT AGA TGG CAT GGT TGG TGT AGG-3′ ), $mGlu_6$ upstream (5′ -CGC GCG ACG CGT GCC GGC TCC GTG CGC CTG GCC GG-3′ ), $mGlu_6$ downstream (GCG GCC GCG CGG CCG CCT ACT TGG CGT CCT CTG CGT TCT C-3′ ) and $mGlu_8$ upstream (5′ -CGC GCG ACG CGT CAA GAG TAT GCC CAT TCC ATC CGG-3′ ), $mGlu_8$ downstream (5′ -GCG GCC GCG CGG CCG CTC AGA TCG AAT GAT TAC TGT AGC TG-3′ ). All constructs were verified by DNA sequencing.

### 2.3. Cell cultures

Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing Glutamax-I and supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml) and 10% dialyzed fetal calf serum (all from Invitrogen, Paisley, UK). In addition, media for mGlu receptor subtypes 1a, 2, 4a, 5a and 6 expressing cell lines contained 1% proline and media for the $mGlu_6$ cell line also contained 0.5 mg/ml G-418. Cells were maintained at 37 °C, 95% humidified air, 5% $CO_2$ in an incubator and subcultured every 3–4 days.

The Chinese hamster ovary (CHO) cell lines stably expressing $mGlu_{1a}$, $mGlu_2$, $mGlu_{4a}$ and $mGlu_{5a}$ have previously been described (Abe et al., 1992; Aramori and Nakanishi, 1992; Tanabe et al., 1992, 1993). A CHO cell line stably expressing $mGlu_6$ was generated as follows. The cDNA coding for rat $mGlu_6$ was transfected into CHO cells using Polyfect according to the manufacturer's instructions (Qiagen, Hilden, Germany). The selection media (5 mg/ml G-418) was added 48 h post-transfection. After 2 weeks of culturing in selection media, single clones were selected by fluorescent immunostaining. The staining protocol was as follows in brief. Cells were blocked with 10% fetal bovine serum in phosphate-buffered saline (PBS). The cells were then incubated for 1 h with anti-myc antibody (1:500; Invitrogen). After washing, cells were incubated for 1 h with secondary Alexa Fluor 488 goat anti-mouse immunoglobulin IgG (1:500; Molecular Probes, Leiden, Netherlands). After a final washing step, fluorescent cell colonies were identified using a Leica DMIL microscope. The selected colonies were amplified in selection media (1 mg/ml G-418) and the final selection was performed based on testing in the cyclic AMP assay.

### 2.4. Measurement of phosphatidylinositol 4,5-biphosphate (PI) hydrolysis and cyclic AMP formation

Both assays were essentially performed as previously described (Hayashi et al., 1992, 1993) with some modifications (Bräuner-Osborne and Krogsgaard-Larsen, 1998). Two days before the assay, 1 million cells were divided into the wells of a 96-well plate. Twenty-four hours before the PI assay, cell media were exchanged with DMEM containing [$^3$H]inositol (2 μCi/ml). On the day of the PI assay, the cells were incubated with ligand dissolved in PBS supplemented with 10 mM LiCl for 20 min, and agonist activity was determined by measurement of the level of $^3$H-labelled mono-, bis- and tris-inositol phosphates by ion-exchange chromatography. On the day of the cyclic AMP assay, cells were preincubated for 20 min in PBS with 1 mM 3-isobutyl-1-methylxanthine (IBMX) and then incubated for 10 min in a similar buffer including 10 μM forskolin and the ligand. The agonist activity was then determined as the inhibitory effect of the forskolin-induced cyclic AMP formation. Cyclic AMP levels were determined by use of a scintillation proximity assay (SPA) according to the manufacturer's protocol (Amersham Biosciences, Uppsala, Sweden).

### 2.5. Binding assays on cloned mGlu receptors

The vectors encoding rat $mGlu_{4a}$ and $mGlu_{8a}$ were transfected into tsA cells (Chahine et al., 1994) using Polyfect as a DNA carrier according to the manufacturer's instructions (Qiagen). Membranes were harvested 2 days after transfection as previously described (Kowal et al., 1998). Protein concentrations were determined using the Bradford kit from Bio-Rad Laboratories (Hercules, USA) with bovine serum albumin as standard. On the day of the assay, membranes were thawed, centrifuged and re-suspended in cold binding buffer.

Binding experiments were performed using the SPA from Amersham, essentially as already described (Monastyrskaia et al., 1999) but with the previously mentioned modifications (Mathiesen et al., 2003). Briefly, the following were placed in a 96-well white optiplate (Packard, Meriden, CT, USA) in a buffer consisting of 30 mM HEPES, 300 mM cholin chloride, 0.1 mM phenylmethylsulfonyl fluoride, pH = 8: membranes (100 μg protein, 50 μl), wheatgerm agglutinin SPA beads (1 mg/well, 50 μl), compounds (100 μl) and [$^3$H]-L-AP4 (30 nM, 50 μl). The plate was sealed and shaken at room temperature for 1 h and counted on a Topcounter (Packard). Non-specific binding was measured in presence of 100 μM L-SOP.

### 2.6. Binding assays at native iGlu receptors

Rat brain membrane preparations used in the receptor binding experiments were prepared according to the method described by Ransom and Stec (1988). Affinities for native AMPA, kainate and NMDA receptors were determined using 5 nM [$^3$H]AMPA (Honore and Nielsen, 1985), 5 nM [$^3$H]kainic acid (Braitman and Coyle, 1987) and 2 nM [$^3$H]GP39653 (Braitman and Coyle, 1987; Honore and Nielsen, 1985; Sills et al., 1991) with some modifications. On the day of experiments, frozen membranes were quickly thawed and homogenised in 40 volumes of ice-cold buffer (pH 7.4) (30 mM Tris–HCl containing 2.5 mM $CaCl_2$, 50 mM Tris–HCl or 50 mM Tris–HCl containing 2.5 mM $CaCl_2$, for [$^3$H]AMPA, [$^3$H]kainate or [$^3$H]CGP39653 binding, respectively), and centrifuged at 48,000 × *g* for 10 min. This step was repeated four times. In [$^3$H]AMPA binding experiments, 100 mM KSCN was added to the buffer during the final wash and during incubation. The final pellet was re-suspended in ice-cold buffer, corresponding to approximately 0.4 − 0.5 mg protein/ml. [$^3$H]AMPA, [$^3$H]kainic acid and [$^3$H]CGP39653 binding were carried out in aliquots consisting of radioligand (25 μl), test solution (25 μl), and membrane suspension (200 μl) and incubated for 30 min, 60 min, and 60 min, respectively. Binding was terminated by filtration through GF/B filters using a 96-well Packard Filter-Mate Cell Harvester and washing with 3 × 250

μl buffer. After drying, 25 μl microscint 0 (Packard) per well was added and the plate was counted on a Topcounter (Packard). Non-specific binding was determined using 1 mM (*S*)-glu.

### 2.7. Cortical slice electrophysiology

A modified rat cortical slice preparation was used for determining the depolarising effects of thioibotenic acid at native receptors (Harrison and Simmonds, 1985; Madsen et al., 1993). Agonists were applied for 90 s. Receptor selectivity was determined by antagonising the responses obtained at agonist concentrations corresponding to their $EC_{50}$ values. NMDA receptor mediated responses could be antagonised with 10 μM 3-(2-carboxy-4-piperazinyl)propyl-1-phosphonic acid (CPP), which was pre-applied for 90 s followed by co-application of agonist and CPP.

### 2.8. Data analysis

Pharmacological experiments were performed in duplicate or triplicate of at least three independent experiments. Single concentration tests were performed in triplicate with two independent experiments. Concentration–response and homologous displacement curves were analyzed by nonlinear regression using GraphPad Prism (GraphPad Software, San Diego, USA). $K_D$ values were estimated using the following equations (Motulsky and Neubig, 1997), where $L$ is the concentration of the radioactive ligand and the $IC_{50}$ was estimated by nonlinear regression from homologous displacement curves: $K_D = IC_{50} - L$. $K_i$ values were calculated from $IC_{50}$ values by use of the Cheng–Prusoff equation (Cheng and Prusoff, 1973).

### 2.9. Convulsant activity

Convulsant activity of the ligands were tested as previously published (Arnt et al., 1995). Male mice (NMRI/BOM, SPF, Bomholtgård, Denmark) weighing 24–26 g were used. The mice were kept in groups of 10 in plastic cages (35 × 30 × 12) in animal rooms at 21 ± 2 °C with a relative humidity of 55 ± 10%, air exchange 16 times per h and a 12-h light/dark cycle (light on 6 a.m. to 6 p.m.). The mice had free access to water and commercial food pellets prior to the study. Ethical permission for the study was granted by the animal welfare committee, appointed by the Danish Ministry of Justice, and all animal procedures were carried out in compliance with the EC Directive 86/609/EEC and the Danish regulations for animal experimentation.

Drug or vehicle was given intracerebroventricularly (i.c.v., 5 μl per mouse over a 30-s period) into the third ventricle, and the animals were observed for presence of clonic or tonic convulsions for the following up to 15 min. The animals were subsequently euthanised by cervical dislocation. The intracerebroventricular administration was performed by means of a 0.5-mm needle connected by polyethylene tubing to a 100-ml Hamilton syringe placed in a micropump (Microject, Carnegie Medicine, Sweden). A piece of plastic catheter allowed for only 3.4 mm of the needle to pass the skull. Skull perforation was performed free-hand by pressing the mouse upwards so that the needle perforated the skull in the midline, 1–2 mm caudal to the eyes, as previously described (Haley and McCormick, 1957). The mouse was removed a few seconds after the infusion was completed. Five mice were used per treatment group.

### 2.10. Homology modelling

Models of the $mGlu_{3-8}$ receptor binding sites were made by comparative protein structure modelling as previously described for the $mGlu_2$ receptor subtype (Clausen et al., 2002). The subsequences of the extracellular ligand-binding domains were submitted to SWISSPROT (Schwede et al., 2000). In constructing the homology models, first approach mode was used, based on a template consisting of a single domain-closed chain of $mGlu_1$ in complex with glu (1EWK.pdb; (Kunishima et al., 2000)). These models were then analysed using methyl and water probes in GRID18 (Goodford, 1985), which assisted adjustment of the positions of glu and crystallographically observed water molecules within the various binding sites.

### 2.11. Docking

The domain-closed monomer from the crystal structure of $mGlu_1$ and homology models of $mGlu_{2-8}$ receptors were refined using Impact2.5/OPLS-AA according to the standard procedure recommended (Schrödinger, Portland, USA) (Jorgensen et al., 1996). The native ligand, tri-ionised glutamate, was included in the refinement process. Grids for docking using Glide 2.5 (Schrödinger) were pre-calculated using a factor 0.9 scaling of van der Waal radii on non-polar atoms. Tri-ionised ibotenic acid and thioibotenic acid were submitted to Monte Carlo analysis using the MMFFs (Merck-Molecular) force field (Halgren, 1999a,b) including Generalized Born/Surface Area (GB-SA) treatment of aqueous solvation in Macromodel 8.1 (Schrödinger). Two minima with similar relative energies were found for both structures: C4–C5–C–H eclipsed (0°) and anti (180°), although MMFFs assigned a slightly lower energy to the eclipsed form of ibotenic acid but to the anti form of thioibotenic acid. For consistency, both ibotenic acid and thioibotenic acid were flexibly docked with Glide to the agonist binding sites of $mGlu_{1-8}$ receptors using the eclipsed form as the input structure.

### 2.12. Ab initio potential energy surface

Since the zwitterions of α-amino acids are generally not minima in gas phase, conformational analysis of the bioac-

tive forms requires solution-phase optimisation. The conformational energies of ibotenic acid and thioibotenic acid in solution, and the internal energies (defined as the gas phase energies at the solution phase geometries) were studied using Density Functional Theory (B3LYP, (Becke, 1993)) with the 6-311+G(d,p) basis set (Krishnan et al., 1980) and Tomasi's polarisable continuum model of aqueous solvation employing the integral equation formalism (IEFPCM, (Cancès and Mennucci, 2001)) in Gaussian'03 (Gaussian, Pittsburg, USA). Starting from optimisations and analytical force constant calculations of the same tri-ionised forms of ibotenic acid and thioibotenic acid in which the C4–C5–C–H dihedral was constrained to 0°, this coordinate was driven through 360° in 30° steps, optimising in solution at each step. Ultrafine integral grids and tight SCF convergence were used throughout, but for numerical reasons, loose criteria were used for geometry convergence. The solution phase partial minima were subjected to single-point gas phase calculations at the same level of theory to give the corresponding internal energies.

## 3. Results

### 3.1. mGlu Receptors

We have previously shown that ibotenic acid is an agonist at both of the group I receptor subtypes ($mGlu_1$/$mGlu_5$) measuring the increase in PI hydrolysis, as well as at the group II subtype $mGlu_2$ measuring the decrease in cAMP formation (Bräuner-Osborne et al., 1998). At the group III mGlu subtypes 4 and 6, however, ibotenic acid had no effect at 1000 μM in the cAMP assay (Table 1; Fig. 2A). Thioibotenic acid was a slightly more potent agonist than ibotenic acid at $mGlu_1$, $mGlu_5$ and $mGlu_2$ receptors (Table 1). By contrast with ibotenic acid, thioibotenic acid was a potent agonist at the group III receptors tested in the cAMP assay. As shown in Table 1 and Fig. 2A, the $EC_{50}$ values of thioibotenic acid at $mGlu_{4a}$ and $mGlu_6$ were 2.6 and 0.96 μM, respectively. We were unable to obtain a solid functional response from transiently expressed $mGlu_8$ receptors, which were consequently characterized in a binding assay as described below.

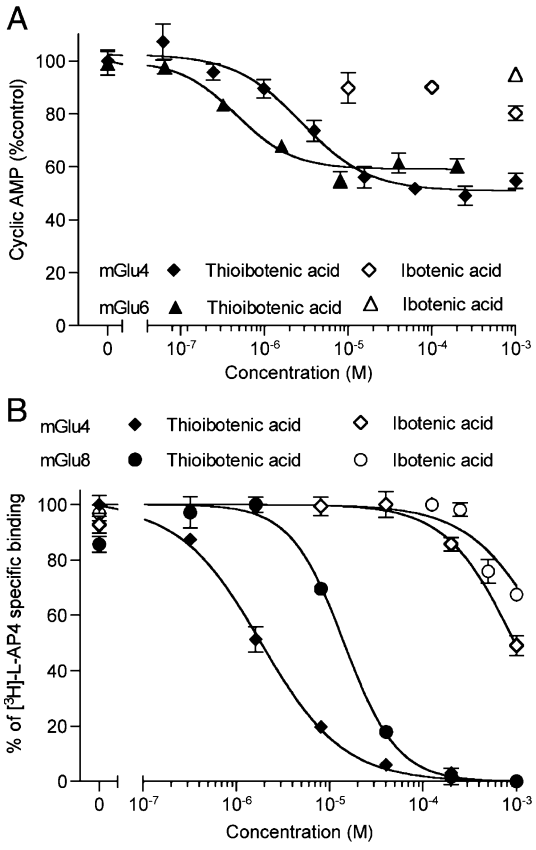


Fig. 2. (A) Concentration–response curves from CHO cells expressing rat $mGlu_{4a}$ and $mGlu_6$. Results are given as % inhibition of 10 μM forskolin-stimulated cyclic AMP production and are from representative experiments performed in triplicate. (B) Concentration-dependent inhibition of [$^3$H]-L-AP4 binding to rat $mGlu_{4a}$ and $mGlu_8$ receptor-expressing cell membranes. Results are expressed as % of [$^3$H]-L-AP4 specific bound and are from representative experiments performed in duplicate.

In order to further investigate this dramatic change in activity at group III mGlu receptors, binding studies were performed. Among the group III mGlu receptors, specific [$^3$H]-L-AP4 binding is only detectable in cells expressing

Table 1
Agonist potencies of ibotenic acid and thioibotenic acid at recombinant rat mGlu receptors and receptor binding affinity to rat $mGlu_{4a}$ and $mGlu_{8a}$ receptor-expressing cell membranes

| | $EC_{50}$ (μM) [$pEC_{50}$ ± S.E.M.] | | | | | $K_i$ (μM) [$pK_i$ ± S.E.M.] | |
|---|---|---|---|---|---|---|---|
| | $mGlu_{1a}$ | $mGlu_{5a}$ | $mGlu_2$ | $mGlu_{4a}$ | $mGlu_6$ | $mGlu_{4a}$ | $mGlu_{8a}$ |
| Ibotenic acid | 43[a] [4.37 ± 0.01] | 17[a] [4.98 ± 0.29] | 110[a] [3.97 ± 0.04] | >1000[a] | >1000 | 820 [3.10 ± 0.07] | >1000 |
| Thioibotenic acid | 12 [4.96 ± 0.11] | 5.4 [5.28 ± 0.07] | 52 [4.30 ± 0.06] | 2.6 [5.63 ± 0.14] | 0.96 [6.10 ± 0.14] | 2.9 [5.59 ± 0.15] | 10 [5.05 ± 0.15] |

Functional data where obtained from CHO cell lines stably expressing the $mGlu_{1, 2, 4, 5 \text{ or } 6}$ receptor subtype. Results on group I mGlu receptors where obtained by measurements of PI hydrolysis and $EC_{50}$ values for groups II and III where determined using the cAMP assay. [$^3$H]-L-AP4 binding experiments were performed on membranes from TSA cells transiently expressing $mGlu_4$ or $mGlu_8$ using the SPA assay. Data are given as mean ± S.E.M. of at least three independent experiments.

[a] Data from (Bräuner-Osborne et al., 1998).

$mGlu_{4a}$ and $mGlu_{8a}$ but not in cells expressing $mGlu_6$ or $mGlu_{7a}$ (Naples and Hampson, 2001). [$^3$H]-L-AP4 binding studies were carried out on rat $mGlu_{4a}$ and $mGlu_{8a}$ receptor-expressing cell membranes using a scintillation proximity assay. No specific [$^3$H]-L-AP4 binding was observed in native tsA cell membranes. The binding data correlate well with the functional data. Ibotenic acid showed very low affinity for $mGlu_{4a}$ ($K_i$ = 820 μM) and no affinity for $mGlu_{8a}$ ($K_i$>1000 μM). Conversely, thioibotenic acid binds to both $mGlu_{4a}$ ($K_i$ = 2.9 μM) and $mGlu_{8a}$ ($K_i$ = 10 μM) group III receptors (Table 1; Fig. 2B).

### 3.2. iGlu receptors

Both ibotenic acid and thioibotenic acid displayed weak affinity for NMDA receptors, with binding affinities ($K_i$) of 5.3 and 13 μM, respectively, in the [$^3$H]CGP39653 binding assay. A slight difference in affinity between the two compounds was observed at kainic acid receptors with ibotenic acid showing low affinity and thioibotenic acid having no affinity (Table 2). Neither compounds showed affinity for the AMPA receptor. In the rat cortical slice model, both ibotenic acid and thioibotenic acid showed functional activity as agonists with $EC_{50}$ values of 9.6 and 25 μM, respectively. These effects were mediated by NMDA receptors as they could be fully antagonised with CPP.

### 3.3. In vivo

We have previously shown that intracerebroventricular administration of NMDA produce convulsions in mice, which can be inhibited by the NMDA antagonist CPP (Arnt et al., 1995). Thioibotenic acid was tested for convulsant effects after intracerebroventricular administration in the same assay. Mice were given either 6 or 12.5 μg thioibotenic acid per animal and both concentrations produced clonic/tonic convulsions. Animals in the control group showed no symptoms.

### 3.4. In silico

The internal energy (gas phase energy at solution phase geometry) and the aqueous phase energy of the tri-ionised (*S*)-forms of the ligands, present at physiological pH and presumed active, were investigated as a function of the dihedral angle between the α-amino acid and the ring. Given the strong polarisability and intra- and intermolecular electrostatic interactions of these ligands, and the exotic heterocyclic ring structures, calculations based on high level quantum mechanics were employed, in conjunction with a continuum solvation model. The potential energy surfaces are summarised in Fig. 3. The global minima in solution have C4–C5–C–H dihedral angles of 0° for both ligands, but deviate sharply in energy at other conformations. The

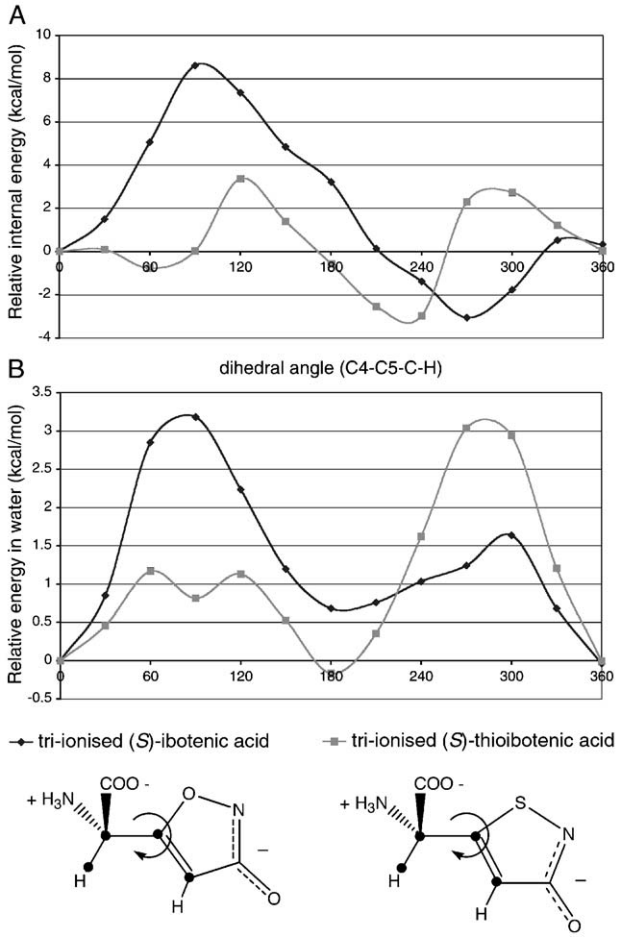


Fig. 3. Conformational energy (kcal/mol) of tri-ionised ibotenic acid (black) and thioibotenic acid (grey) as a function of C4–C5–C–H dihedral angle in water (IEFPCM model) at B3LYP/6-311 + G(d,p). (A) Internal gas phase conformational energy penalty at solution phase geometry. (B) Energy in solution. Ibotenic acid displays a substantial penalty at around 90°.

Table 2
Receptor binding affinity to iGlu receptors in rat cortical membranes and agonist effect in the rat cortical slice model

| | $K_i$ (μM) [p$K_i$ ± S.E.M.] | $IC_{50}$ (μM) [p$IC_{50}$ ± S.E.M.] | | $EC_{50}$ (μM) [p$EC_{50}$ ± S.E.M.] |
|---|---|---|---|---|
| | [$^3$H]CGP39653 | [$^3$H]AMPA | [$^3$H]Kainic acid | Slice model |
| Ibotenic acid | 5.3 [5.28 ± 0.04] | >100 | 22 [4.66 ± 0.07] | 9.6[a,b] |
| Thioibotenic acid | 13 [4.88 ± 0.02] | >100 | >100 | 25 [4.63 ± 0.07][b] |

Data are given as means ± S.E.M of at least three independent experiments.
[a] Data from (Madsen et al., 1998).
[b] The effect is NMDA receptor mediated as it can be antagonised by 10 μM CPP.

published crystal structure of ibotenic acid has a dihedral angle of around 240°, in agreement with a modest energetic advantage (ca. 1 kcal $mol^{-1}$) upon condensation compared to the global minimum in solution. With every 1.4 kcal/mol internal energy costing a 10-fold decrease in affinity, thioibotenic acid is conformationally versatile; only regions close to 120° and 270–300° are energetically unfavorable. By contrast, ibotenic acid cannot bind in a broad range of conformations from approximately 60–180°.

We have previously demonstrated that docking to these homology models, and specifically $mGlu_2$, can successfully give credible binding modes and reproduce selectivity profiles matching those of subtype-selective ligands, including the group II selective ligand (−)-2-oxa-4-aminobicyclo[3.1.0]hexane-4,6-dicarboxylate (LY379268) (Clausen et al., 2002). In order to further validate the reliability of our models, we docked LY379268 to $mGlu_1$ and $mGlu_4$, representing group I and group III, respectively. We obtained a rank order of docking scores (E-model) of $mGlu_2$>$mGlu_4$>$mGlu_1$, which is in excellent agreement with the published pharmacological activities (Monn et al., 1999). Moreover, for this ligand, Glide completely fails to find ($mGlu_1$) or finds only at the cost of lowered scoring and pose ranking ($mGlu_4$) a glutamate-like binding mode at

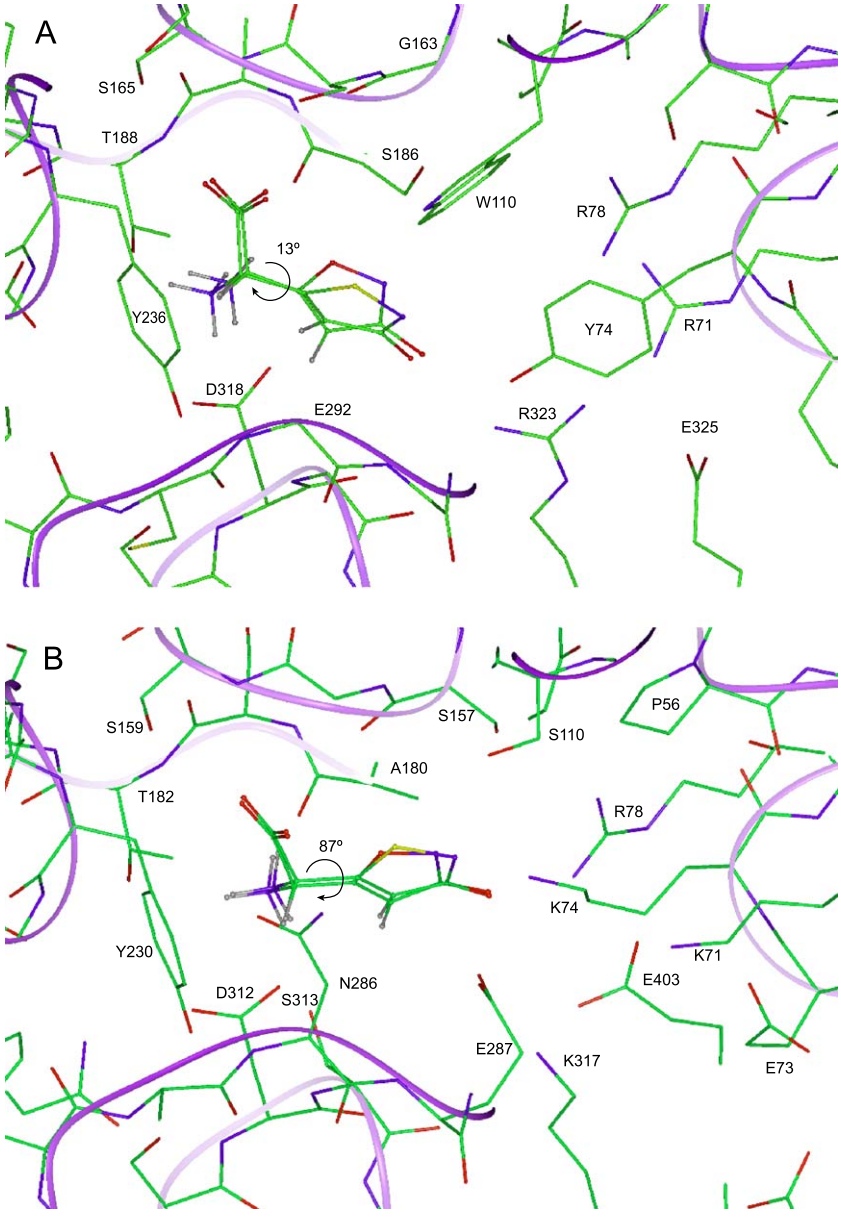


Fig. 4. Ibotenic acid and thioibotenic acid docked to (A) the crystal structure of the $mGlu_1$ receptor and (B) a homology model of the $mGlu_4$ receptor binding site. Dihedral angle (C4–C5–C–H) marked for ibotenic acid. Group I mGlu receptors allow a dihedral angle close to 0° while group III mGlu receptors require a dihedral angle close to 90°.

those receptors the ligand does not prefer. Docking ibotenic acid and thioibotenic acid to all eight mGlu subtypes produced a clear difference in preferred binding modes between group III receptors and receptors from groups I and II receptors. Fig. 4A and B presents the ligands docked to the crystal structure of the domain-closed binding site of $mGlu_1$ and a homology model of $mGlu_4$. According to the docked poses with the best energy scores, dihedral angles close to zero are preferred at group I receptors, and small positive angles are preferred at group II receptors, in part due to a $\pi-\pi$ stacking arrangement with TYR144/150 ($mGlu_{2/3}$). The steric and electronic environment of group III binding sites appears to require a dihedral angle closer to 90° in order to meet the hydrogen bonding requirements of both the proximal and distal ionised groups.

## 4. Discussion

Thioibotenic acid activates the NMDA receptors, which are known to have an excitatory effect on the CNS and to induce convulsions in vivo (Bischoff et al., 1995; Krogsgaard-Larsen et al., 1980; Madsen et al., 2001). On the other hand, thioibotenic acid also activates group III mGlu receptors, which have been shown to be neuroprotective against iGlu receptor induced excitotoxicity (Bruno et al., 2000; Henrich-Noack et al., 2000). In order to investigate whether the putative neuroprotective effects of thioibotenic acid mediated by group III mGlu activation could inhibit its neurotoxic effects arising from iGlu activation, thioibotenic acid was tested in vivo. However, the neurotoxic effect attributable to iGlu activation apparently was stronger than the neuroprotective effect of group III mGlu activation, since a dose of 6 μmol per mice administrated intracerebroventricularly induced convulsions.

The difference in pharmacology between ibotenic acid and thioibotenic acid at group III mGlu receptors is remarkable and all the more intriguing because of the similarity of structure and pharmacological profiles of these two heterocyclic glu analogues at other glu receptors. Ibotenic acid is inactive, while thioibotenic acid ($EC_{50}=2.6$ μM) is quite potent at $mGlu_4$ as compared to glu ($EC_{50}=12$ μM) and the standard compound L-AP4 ($EC_{50}=0.91$ μM) (Bräuner-Osborne and Krogsgaard-Larsen, 1998). The differences in potency between the two title compounds at $mGlu_4$ and $mGlu_6$ are at least 400- and 1000-fold, respectively. Furthermore, binding data at $mGlu_4$ show that it is indeed a primary difference in binding affinity rather than a difference in, e.g., receptor activation, indicating a difference in binding energy of 3–4 kcal $mol^{-1}$. Finally, the $mGlu_8$ binding data show that this group III receptor displays the same preference. This is the first occasion on which we have observed such striking differences in pharmacological activity arising from this atomic replacement. Such a large binding energy difference ought to have an obvious cause—typically seen for example when extra steric bulk on a ligand clashes with a receptor-essential volume, abolishing activity. However, it is the slightly larger, more hydrophobic isothiazole which retains activity, effectively ruling out steric causes. Along similar lines, a differentially unfavorable ligand-receptor electrostatic contact also seems unlikely, as the approach of an electronegative receptor atom towards the ring S or O is unfavorable in both cases. Abundant examples of isoxazole and isothiazole glu analogues binding with $K_i$ values of a similar order of magnitude, rule out differences in ligand p*K*a or solvation effects as the cause. The only possible remaining source of selectivity is the internal energy of the ligand in response to the receptor environment, compared with the conformational ensemble in solution, according to the criteria established by Boström et al. (1998). We therefore investigated the possibility of a change in preferred binding mode at group III mGlu receptors compared to groups I and II, requiring ibotenic acid to meet an energetically impossible conformation were it to make the necessary polar interactions with the receptor, but still allowing thioibotenic acid to bind. Numerous changes in the charged residues around the distal anionic group between mGlu subtypes give ample possibility for such a change in binding mode. Conformational analysis reveals that at dihedral angles between 60° and 120°, the internal energies deviate sharply by the required 3–4 kcal/mol in favour of thioibotenic acid, due in part to electrostatic repulsion between the α-carboxylate and the ring oxygen of ibotenic acid. The docking study predicts that for this type of ligand, conformations with dihedral angles close to 90° are indeed preferred for binding to group III mGlu receptors, at which point thioibotenic acid has no significant conformational energy penalty while ibotenic acid shows an energy maximum, strongly supporting this hypothesis. Whether or not other ligands, including glu, adopt a slightly different binding mode at group III mGlu receptors compared to groups I and II remain to be demonstrated. Such a difference in the preferred orientation of the distal anion could potentially be exploited in the design of subtype-specific ligands. We are currently engaged in mutation and docking studies on group III receptors in order to further characterise the basis of selectivity at these receptors.

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